AD_____

Award Number: DAMD17-99-1-9214

TITLE: Differential Processing of Cyclin E Variants in Normal Versus Tumor Cells and Their Role in Breast Cancer Oncogenesis

PRINCIPAL INVESTIGATOR: Mollianne J. McGahren
Khandan Keyomarsi, Ph.D.

CONTRACTING ORGANIZATION: The University of Texas
M. D. Anderson Cancer Center
Houston, Texas 77030

REPORT DATE: September 2002

TYPE OF REPORT: Annual Summary

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	September 2002	Annual Summary (1 Sep 01 - 31 Aug 02)

4. TITLE AND SUBTITLE
Differential Processing of Cyclin E Variants in Normal Versus Tumor Cells and Their Role in Breast Cancer Oncogenesis

5. FUNDING NUMBERS
DAMD17-99-1-9214

6. AUTHOR(S):
Mollianne J. McGahren
Khandan Keyomarsi, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
The University of Texas
M. D. Anderson Cancer Center
Houston, Texas 77030
E-Mail: mollianne.j.mcgahren@uth.tmc.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES
report contains color

12a. DISTRIBUTION / AVAILABILITY STATEMENT
Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*

Cyclin E is a positive regulator, which controls the transition of the G1 to S phase of the cell cycle. When associated with CDK2, it is responsible for cells passing through the restriction point, which is the barrier between G1 and S. This commits the cell to complete one round of cell division. Previous findings by this laboratory have found that overexpression of cyclin E and the presence of lower molecular weight isoforms (LMW) are found more often in breast tumors and cancer cell lines when compared to normal tissues and cells. Also, tumor cells, but not normal cells have the mechanisms to proteolytically cleave the full length cyclin E into these LMW forms. An altered cyclin E may contribute to the deregulation of the G1 to S checkpoint and lead to tumorigenesis. Our laboratory has also identified through mutational and biochemical analysis, the region of cyclin E that is proteolyticaly cleaved to generate the LMW forms. Epitope tagged (i.e. FLAG) plasmid constructs have been generated which contain either the full length cyclin E (EL) or the LMW isoforms (T1 and T2) found in tumor cells. Each of these three constructs were stably transfected into immortalized mammary epithelial cells, 76NE6 cells. Our results show that the LMW constructs are biologically functional and their overexpression in 76NE6 cells increases the cells ability to enter S and G2/M phase by a 2-fold increase over vector alone cells. This was not the case with the overexpression of EL, the form naturally expressed in normal cells. We also show that 76NE6 cells which overexpress either T1 or T2 have a lower level of p27 as compared to cells overexpressing the EL form. These results suggests that deregulation of the cell cycle and altered substrate specificity of cyclin E LMW forms contributes to the oncogenesis process.

14. SUBJECT TERMS	15. NUMBER OF PAGES
cyclin E, breast cancer, cell cycle	12
	16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction:

One of the first steps in the multi-step process of tumorigenesis is the deregulation of the cell cycle, which can cause the cells to replicate uncontrollably. Many cancers have been associated with the abnormal expression of proteins involved in the regulation of the cell cycle. Alterations of cyclin E, a positive regulator of the G1 to S phase transition, have been found in several types of cancer, including breast carcinomas. Furthermore, in breast cancer patients, there is a correlation with overexpression of cyclin E and the lower molecular weight (LMW) forms and poor patient prognosis. To date, there is no direct evidence for the LMW forms role in tumorigenensis; however, the finding that these LMW forms are only present in tumor cells and not normal cells, along with our preliminary data on these LMW forms, leads us to believe they could play a role in the tumorigenic process. Characterizing the effects of the overexpression of these LMW forms will address their role if any, in breast cancer tumorigenisis.

Body:

We initiated our characterization of the lower molecular weight (LMW) isoforms of cyclin E by examining their appearance in normal versus tumor tissues and breast tumor tissues. Figure 1 is a representative western blot for cyclin E, cyclin D1 and PCNA for 10 patients with different stages of breast cancer. As illustrated, the tumor cell line MDA-MB-157, and human breast tumor tissues exhibit an altered cyclin E expression. Cyclin E antibody detected not only the 50 kDa form but also the lower molecular weight isoforms, which are characteristic of the stage of the disease, while normal cells only expressed the 50 kDa, full length, form of cyclin Lanes 2, 6, and 10 correspond to tumors that overexpressed LMW forms of cyclin E and all three of these patients died of disease. These analysis suggest that the lower molecular weight isoforms of cyclin are only expressed in tumor cells and that such overexpression of these forms is indicative of the stage of the disease.

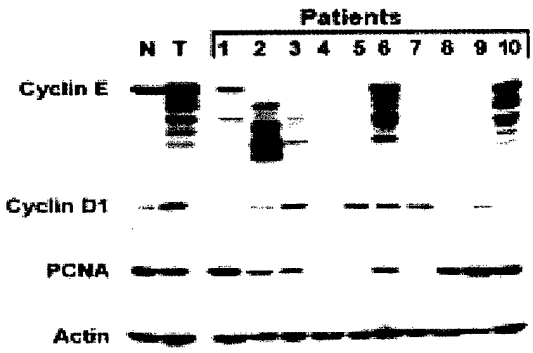

Figure 1: Western blot analysis showing an altered cyclin E expression in tumor cells. Whole-cell lysates were extracted from 10 breast cancer tissues obtained from patients whose diagnosis was infiltrating ductal carcinoma. The clinical staging, (i.e. TNM) and outcome (1: Dead due to disease or relapse of cancer; 0, no evidence of disease) are as follows: 1: I,0; 2: IIB,1; 3: IIA,0; 4: IIA,0; 5: I,0; 6: IIIB,1; 7: I,0; 8: IIA,0; 9: IIB,0; 10: IIB,1. Protein extracts were analyzed on Western blots (50 μg of protein extract/lane) and hybridized with the indicated antibodies. The control lanes correspond to cultured normal (N) and tumor (T) cell lines, where N=76N normal cell strain and T=MDA-MB-157 tumor cell line.

To determine the role of the LMW forms of cyclin E in tumorigenesis , we transfected an immortalized (non-tumorigenic) breast cell line, 76NE6, with the full length (EL) and the LMW forms of cyclin E (T1 and T2). 76NE6 cells are derived from a parental cell line, 76N, which were obtained from reduction mammoplasty and have been immortalized by transfection by the plasmid 16E6 gene of the HPV genome (1). The E6 proteins of HPVs bind indirectly to p53 mediating its degradation (2). Therefore, the immortal 76NE6 cells have lost their normal p53 phenotype. However, their Rb pathway is intact and therefore they have not lost their ability to arrest at the G1/S restriction point (3). Furthermore, 76NE6 cells do not express the LMW forms of cyclin E and do not have the machinery to process the full length cyclin E in to the LMW forms (4).

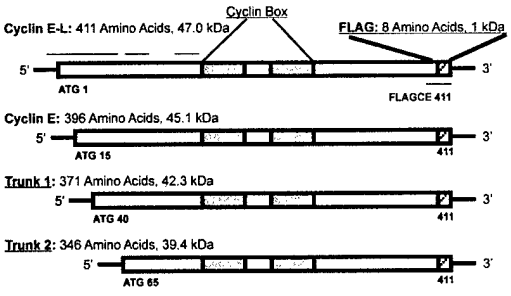

Figure 2: Generation of cyclin E-FLAG constructs. Schematic representation of different FLAG-tagged cyclin E constructs. The FLAG tag regions is shown as a hatched box and the cyclin box (CDK binding) region is shown as shaded boxes.

We generated stable pools of 76NE6 cells expressing either the pcDNA4.0 vector (containing the gene for zeocin resistance) alone, or vector harboring the, T1-FLAG, or T2-FLAG (T1 and T2 encompass the LMW forms of cyclin E, see figure 2). The constructs were then linearized by restriction enzyme digestion and transfected in to the76NE6 cells using Fugene (Roche). After 18 hours, the transfected cells were harvested and

subjected to western blot analysis with antibodies to cyclin E and FLAG to determine transient expression of the protein product of the transfected DNA. Transient expression was high in all 3 experimental constructs (data not shown). Therefore, stable transfections were initiated by maintaining cells in 10μg/mL zeocin. Pools of clones have undergone several passages of selection in zeocin and have been harvested. From these pools, cells were harvested and sonicated to generate whole cell lysate for analysis by western blotting (Figure 3).

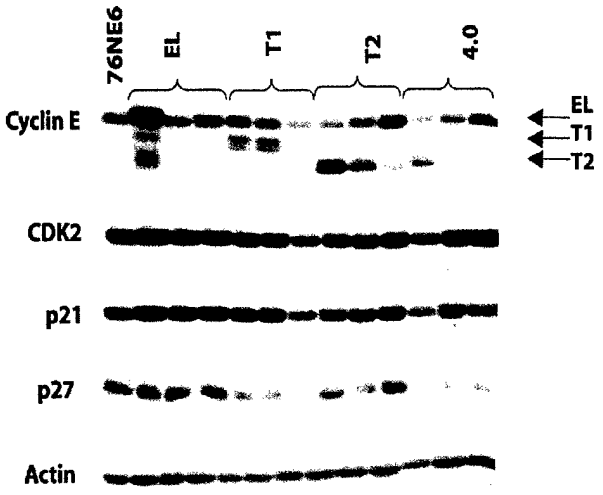

Figure 3: Stable pools of 76NE6 overexpressing the LMW forms of cyclin E. Cyclin EL, cyclin E- T1, or cyclin E-T2 constructs were transfected into 76NE6 cells using Fugune (See text for methods). The figure depicts western blot analysis of the stable pools with the indicated antibodies

Western blot analysis of stable pools transfected with each of the different forms of cyclin E revealed that we have several pools overexpressing the LMW forms, all of which are a result of the transfection since the parental 76NE6 cells (first lane) do not express the LMW forms of cyclin E. Interestingly, there is some evidence that overexpression of the T2-form of cyclin E results in a decrease in the levels of the full length, wild type form of cyclin E, possibly indicating a role for the LMW forms in further proteolytic cleavage or degradation of the full length cyclin E. We also find that overexpression of the LMW forms of cyclin E results in decreased expression of p27, as compared to the full length stable pools or the parental. There were no significant changes in p21 expression in the presence of the LMW forms of cyclin E. It will be necessary to determine whether there are differences in the amount of the CKIs and CDK2 that actually interact with the various forms of cyclin E by immunoprecipitation (IP) western, and whether these complexes are active by IP kinase assays. From these experiments we will be able to determine whether the increased levels of cyclin E are in complex with their partner, CDK2, causing the increased phosphorlyation of pRb which could result in progression through the G1 checkpoint.

We next examined the cell cycle progression of the transfected stable pools by staining the cells with propidium iodide (PI) and analyzing them by flow cytometry for DNA content (Fig 4A & 4B). The results reveal that the LMW forms of cyclin E lead to significant progression of the cells from G1 phase to S phase. The LMW forms appear to be mitogenic, stimulating the cells in to S phase up to 2.5-fold over the empty vector. Meanwhile, there are 50% less cells in G1 in cells transfected with the LMW forms as compared to the empty vector. Interestingly, the full-length form of cyclin E showed a slight decrease in S phase, and a concomitant increase in G1 as compared to the empty vector. This was evident in both the transfectant populations we analyzed (Fig 4B panels). These data suggest than the overexpression of the LMW forms, but not the full length form, of cyclin E have a stimulatory effect, causing the cells to progress through the cell cycle. Therefore the cells expressing the LMW forms of cyclin E appear to have aberrant control of the most critical checkpoint in the cell cycle.

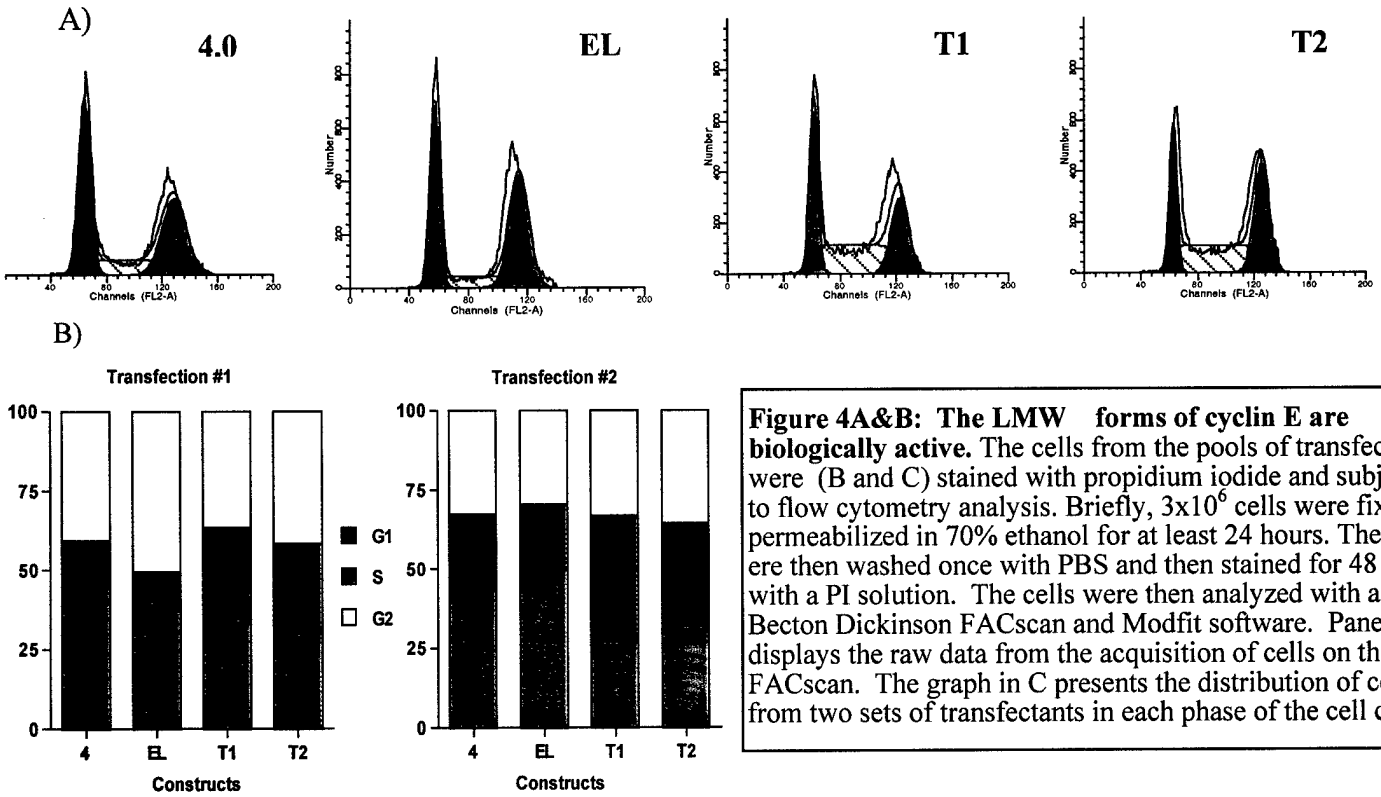

Figure 4A&B: The LMW forms of cyclin E are biologically active. The cells from the pools of transfectants were (B and C) stained with propidium iodide and subjected to flow cytometry analysis. Briefly, $3x10^6$ cells were fixed and permeabilized in 70% ethanol for at least 24 hours. The cells ere then washed once with PBS and then stained for 48 hours with a PI solution. The cells were then analyzed with a Becton Dickinson FACscan and Modfit software. Panel C displays the raw data from the acquisition of cells on the FACscan. The graph in C presents the distribution of cells from two sets of transfectants in each phase of the cell cycle.

A growth curve analysis was performed on each of the transfected pools to determine whether the observed increased progression through the cell cycle by the transfectants expressing the LMW isoforms correlated with a proliferative advantage for these cells compared to those transfected with the full length cyclin E or the untransfected controls (Fig 5). Our results show that the cells transfected with either of the LMW isoforms of cyclin E have a significantly faster rate of proliferation than the cells transfected with the full length of cyclin E or the empty vector. The cells transfected with the LMW isoforms of cyclin E had approximately 5-fold increase in cell count over the empty vector control. Cells transfected with EL do not show a significant difference from the vector alone control. Additionally, the cells from the EL tansfected pool plateau when they reach confluence, while cells transfected with either of the LMW forms proliferated to a much higher density before they reached a plateau, clearly demonstrating a resistance to contact inhibition. These data suggest that the LMW isorms of cyclin E are involved in the deregulation of the cell cycle resulting in increased cell proliferation and resistance to contact inhibition characteristic of a transformed phenotype.

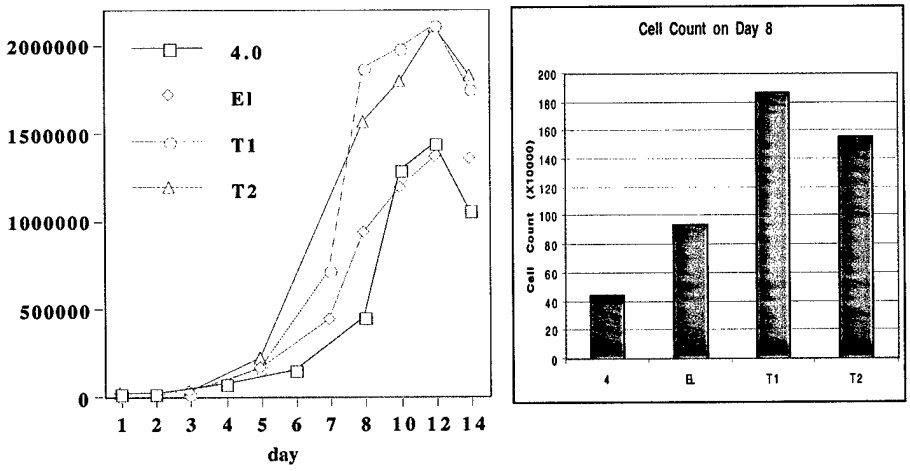

Figure 5: The LMW isoforms of cyclin E provide a growth advantage to the 76NE6 cells. $1X10^4$ Cells from the pools of transfectants were plated in to 6 well plates. (a) Cells were harvested and counted in triplicates atleast every 48 hours and counted using a Coulter Counter. (b) Bar graph representing the difference in cell count on day 8 of the growth curve.

In addition, soft agar colony forming assays are in the preliminary stages. Although there is no data to be shown yet, initial microscopic analysis provides evidence that cells from the pools of transfectants expressing the LMW isoforms of cyclin E have the ability to form colonies in the soft agar more readily than those transfected with the full length cyclin E or the empty vector. Furthermore, plating efficiency studies were performed to observe growth characteristics of the transfected cells. Briefly, cells were plated at densities of 500, 1000 and 5000 cells per 100mm tissue culture dish and observed for colony formation and morphological changes. While the cells transfected with the LMW isoforms did appear to have both increased colony formation and proliferation compared to the EL or empty vector transfected cells, the most intriguing observations were made of the differences in morphology (data not shown). The colonies formed by the EL or empty vector transfected cells were well defined and the cells were homogeneous in size and shape. However, colonies of the LMW isoform transfected cells contained a more heterogeneous population of cells including irregularly shaped cells and larger cells encompassing several nuclei. This may support recent findings linking cyclin E overexpression to genomic instability and add to the rationale for studying the role of cyclin E in tumor progression.

Key Research Accomplishments:

- We have several clones of the full length cyclin E as well as the lower molecular weight (LMW) isoforms to begin to study the difference in processing of cyclin E between normal and tumor cells. Instead of using the retovirus and the tetracycline inducible vector, pBSTR-1, we are going to pick clones that have different expression levels of cyclin E, both full length and LMW isoforms ,and compare these clones by western blot, immunoprecipitation (IP), flow cytometry, growth curves and soft agar assays.
- We have begun to analyze these LMW isoforms by western blot—to look at expression level, growth curves—to evaluate what changes the LMW isoforms have on cell growth, soft agar assays—to look at phenotypic changes, and flow cytometry—to look at the changes in cell cycle and their progression through the cell cycle and the effect the LMW isoforms have on the cell cycle. IP still needs to be performed on the varying clones.

Reportable Outcomes:

Research

Manuscripts

- Harwell RM, Porter DC, Danes C, et al. 2000. Processing of cyclin E differs between normal and breast cells. *Cancer Research* 60:481-489.
- Porter DC, Zhang N, Danes C, McGahren M, et al. 2001. Tumor-specific proteolytic processing of cyclin E generates hyperactive lower-molecular-weight forms. *Mol. Cell. Bio.* 21:6254-6269.

Abstract

- Lavu H, Harwell RM, Danes C, et. al. Clonal variants of MCF-10A cells have a deregulated cell cycle. *Proceed. Amer. Assoc. Can. Res.* 41:2317.

Conclusions:

The lower molecular weight (LWM) isoforms of cyclin are only expressed in tumor cells and such overexpression of these forms is indicative of the stage of the disease. Cyclin E could be used as a strong prognostic indicator of correlation with overexpression of cyclin E and the LMW forms and poor patient prognosis. Furthermore, there is evidence that overexpression of the T2-form of cyclin E results in a decrease in the levels of the full length, wild type form of cyclin E, possibly indicating a role for the LMW forms in further proteolytic cleavage or degradation of the full length cyclin E. We also find that overexpression of the LMW forms of cyclin E results in decreased expression of p27, as compared to the full length stable pools or the parental. By performing immunoprecipitation (IP) assays, we will be able to determine whether there are differences in the amount of the CKIs and CDK2 that actually interact with the various forms of cyclin E by IP western, and whether these complexes are active by IP kinase assays. From these experiments we will be able to determine whether the increased levels of cyclin E are in complex with their partner, CDK2, causing the increased phosphorlyation of pRb which could result in progression through the G1 checkpoint. Overexpression of the LMW forms, but not the full length form, of cyclin E have a stimulatory effect, causing the cells to progress through the cell cycle. Therefore the cells expressing the LMW forms of cyclin E appear to have aberrant control of the most critical checkpoint in the cell cycle. Finaly, data suggests that the LMW isoforms of cyclin E are involved in the deregulation of the cell cycle, resulting in increased cell proliferation and resistance to contact inhibition characteristic of a transformed phenotype. Since cycllin E is one of the most critical regulators of the G1 to S transition, it's deregulation by the LMW isoforms may be a critical step in tumorigenesis. Taken together, this may support recent findings linking cyclin E overexpression to genomic instability and add to the rationale for studying the role of cyclin E in tumor progression.

References:

1. Band, V., Zajchowski, D., Kulesa, V., and Sager, R. Human papilloma virus DNAs immortalize normal human mammary epithelial cells and reduce their growth factor requirements, Proc Natl Acad Sci U S A. *87:* 463-7., 1990.
2. Scheffner, M., Werness, B. A., Huibregtse, J. M., Levine, A. J., and Howley, P. M. The E6 oncoprotein encoded by human papillomavirus types 16 and 18 promotes the degradation of p53, Cell. *63:* 1129-36., 1990.
3. Band, V., De Caprio, J. A., Delmolino, L., Kulesa, V., and Sager, R. Loss of p53 protein in human papillomavirus type 16 E6-immortalized human mammary epithelial cells, J Virol. *65:* 6671-6., 1991.
4. Porter, D. C., Zhang, N., Danes, C., McGahren, M. J., Harwell, R. M., Faruki, S., and Keyomarsi, K. Tumor-specific proteolytic processing of cyclin E generates hyperactive lower-molecular-weight forms, Mol Cell Biol. *21:* 6254-69., 2001.